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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 :
C07C 103/52, C07G 7/00
A61K 39/00, G01N 21/00

A1

(11) International Publication Number: WO 85/ 04654

(43) International Publication Date: 24 October 1985 (24.10.85)

(21) International Application Number: PCT/US85/00565

(22) International Filing Date: 4 April 1985 (04.04.85)

(31) Priority Application Number: 597,434

(32) Priority Date: 6 April 1984 (06.04.84)

(33) Priority Country: US

(60) Parent Application or Grant
(63) Related by Continuation
US 597,434 (CIP)
Filed on 6 April 1984 (06.04.84)

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: A PEPTIDE VACCINE OR DIAGNOSTIC, AND A POLYPEPTIDE USEFUL THEREFOR

(57) Abstract

A polypeptide that is smaller than a naturally-occuring gonococcal pilin protein but is capable of immunological mimicking a conserved antigenic determinant site within a variable region within the carboxy-terminal half of a gonococcal pilin Vaccines, inocula, methods of immunization, and diagnostic assays utilizing such a polypeptide as well as antibodies raised by the polypeptide are disclosed.

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A PEPTIDE VACCINE OR DIAGNOSTIC, AND A POLYPEPTIDE USEFUL THEREFOR

Description

Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Serial No. 597,434, filed on 6 April 1984.

Technical Field

This invention relates to antigens, immunogens, and to vaccines utilizing such immunogens. More particularly, this invention relates to polypeptide antigens or immunogens, antibodies raised by such immunogens, and a vaccine suitable for the prevention of gonorrhea.

Background Art

The annual incidence of reported infections by Neisseria gonorrhoeae is estimated to be about two million cases. A gonococcal infection in men usually results in a relatively uncomplicated urogenital infection. Disseminated gonococcal infection is reported to occur in 1 to 3% of those with gonorrhea, but the morbidity of this disease with current therapy is slight.

On the other hand, in women infected with gonorrhea, salpingitis occurs in 10 to 20%, and even when adequately treated, may result in recurrent salpingitis, ectopic pregnancy, and infertility. It is estimated that salpingitis leads to 1.8 million office visits to private physicians and 220,000 hospitalizations each year in the United States.

The control of gonorrhea by public health measures to date has been difficult. A vaccine composed of whole, killed gonococci has not been efficacious, Greenburg et al., "Preliminary Studies On The Development Of A Gonococcal Vaccine," Bull. Wld. Hlth. Org. 45:531 (1971). Penicillinase-producing strains of gonococci have also emerged.

With a view toward developing an efficacious vaccine, several studies have examined surface components of the gonococcus, including lipopolysaccharide (LPS), peptideglycan, outer membrane proteins, capsules, IgA1 protease, and pili. Of the foregoing, pili have been proposed as essential constituents of a gonorrhea vaccine, and it has been reported that pili are immunogenic and nontoxic for humans. See, generally, Schoolnik et al., "A Pilus Peptide Vaccine For The Prevention Of Gonorrhea," Prog. Allergy 33:314-331 (1983).

The pili are proteinaceous surface appendages of bacteria which promote infectivity by facilitating the attachment of the bacterial cell to host epithelial tissues. In the case of N. gonorrhoeae, the pilus protein is a primary surface antigen. Each pilus is a fimbriak structure composed of repeating identical subunits (pilin), each having a molecular weight of approximately 18,000 daltons. There are many serotypes of N. gonorrhoeae pili; however, peptide mapping studies of antigenically and immunogenically different pilin indicate that they all share a conserved region located at the amino terminal end of the protein. On the other hand, the carboxy terminal region of the protein has been found to be variable from serotype to serotype. A general description of the pilus protein can be found in Meyer et al., Cell 30:45:52 (1982).

Schoolnik et al., op. cit., propose an immunogenic pilus peptide that encompasses the conserved region at the amino terminal end of the protein. However, to date, an effective vaccine has not resulted from that particular approach.

In contradistinction to the foregoing, it has now been found that the variable region of N. gonorrhoeae pilin protein near the carboxy terminal

end of the protein contains both hypervariable sequences as well as conserved sequences defining antigenic determinant sites that can be utilized to prepare antigens and/or immunogens that immunologically mimick these antigenic determinant sites, and that elicit antibody production in a mammalian host.

Summary Of The Invention

The present invention contemplates a polypeptide that is smaller than a naturally-occurring gonococcal pilin protein, and the pharmaceutically acceptable salts thereof, that are capable of immunologically mimicking a conserved antigenic determinant site within a variable region of the carboxy-terminal half of the gonococcal pilin and thus are capable of being an antigen, or an immunogen, against a gonococcal infection. Additionally, the present invention contemplates a vaccine that contains such an immunogen as well as a method of immunization against a gonococcal infection. This invention further contemplates a diagnostic assay utilizing a polypeptide of this invention and/or a receptor such as an antibody elicited by such a polypeptide.

The polypeptide of this invention comprises at least one amino acid residue sequence, containing at least five amino acid residues and up to about 60 amino acid residues, that defines a sequence capable of immunologically mimicking an antigenic determinant site of a gonococcal pilin. This amino acid residue sequence can repeat as a unit one or more times in the same polypeptide molecule. More than one type of such repeating unit, and more than one repeating unit of the same type, can be present in a single polypeptide molecule that embodies the present invention.

Such polypeptide can be made as a fusion protein synthesized by genetic engineering techniques or it can be built-up from individual amino acid residues, or amino acid residue blocks.

Polypeptides embodying this invention can be defined as including the amino acid residue sequence, taken from left to right and in the direction from the amino-terminus to the carboxy-terminus, of the formula

$$-X^1-X^2-X^3-X^4-X^5-$$

wherein X^1 is an amino acid residue having a positively charged side chain and is a member of the group consisting of histidine (HIS), lysine (LYS) and arginine (ARG), X^2 and X^3 can be the same or different and are non-polar amino acid residues that are members of the respective groups consisting of leucine (LEU), proline (PRO), tryptophan (TRP), phenylalanine (PHE), valine (VAL), alanine (ALA) and isoleucine (ILE), and X^4 and X^5 can be the same or different and are polar but uncharged amino acid residues that are members of the respective groups consisting of serine (SER), threonine (THR), cysteine (CYS) and glycine (GLY).

Preferred amino acid residue sequences that define a desired antigenic determinant site are included in the sequences, taken from left to right and in the direction from the amino-terminus to the carboxy-terminus, of the formula:

-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-;

-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-LYS-ALA-SER-ASP-ALA-LYS-; and

-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-PRO-VAL-THR-ARG-,

and antigenically related variants thereof. Also preferred are the corresponding polypeptides themselves, that is,

H-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-OH;

H-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-LYS-ALA-SER-ASP-ALA-LYS-OH; and

H-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-PRO-VAL-THR-ARG-OH,

the pharmacologically acceptable salts thereof, and antigenically related variants thereof.

Detailed Description Of Preferred Embodiments

Polypeptides of the present invention are smaller than the naturally-occurring gonococcal pilin protein and include an amino acid residue sequence of at least five to about 60 amino acid residues, preferably five to twenty amino acid residues, that immunologically mimicks a conserved antigenic determinant site in the variable region of the carboxy-terminal half of gonococcal pilin protein. As such, the present polypeptides are useful by themselves, or as pharmaceutically acceptable salts, as the active constituent in a vaccine, as an inoculum, or in a diagnostic assay.

The term "antigenic determinant," as used herein, designates the structural component of a molecule that is responsible for specific interaction with corresponding antibody (immunoglobulin) molecules elicited by the same or related antigen. Antigenic determinants in the present polypeptides comprise chemically active surface groupings of amino acid residues.

The term "antigen," as used herein, means an entity that is bound by an antibody.

The term "immunogen," as used herein, describes an entity that induces antibody production in the host animal. In some instances the antigen and the immunogen are the same entity, while in other instances the two entities are different.

The phrase "immunologically mimicks" is used herein to mean that an immunogenic polypeptide of this invention is not a natural protein or a cleaved fragment of a natural protein, but a manufactured polypeptide, as by solid phase synthesis or genetic engineering techniques, which polypeptide induces production of antibodies that bind to the inducing polypeptide and also to a corresponding pilin or pilin polypeptide portion.

All amino acid residues identified herein are in the natural or L-configuration unless otherwise specified. In keeping with standard peptide nomenclature, abbreviations for amino acid residues that have been used herein are as follows:

Symbol		Amino Acid
1-Letter	3-Letter	
Y	TYR	-L-tyrosine
G	GLY	-glycine
F	PHE	-L-phenylalanine
M	MET	-L-methionine
A	ALA	-L-alanine
S	SER	-L-serine
I	ILE	-L-isoleucine
L	LEU	-L-leucine
T	THR	-L-threonine
V	VAL	-L-valine
P	PRO	-L-proline
K	LYS	-L-lysine
N	ASN	-L-asparagine
H	HIS	-L-histidine
Q	GLN	-L-glutamine
E	GLU	-glutamic acid
W	TRP	-L-tryptophan
R	Arg	-L-arginine
D	ASP	-L-aspartic acid
C	CYS	-L-cysteine.

The term "pharmaceutically acceptable salts," as used herein, refers to the non-toxic alkali metal, alkaline earth metal and ammonium salts commonly used in the pharmaceutical industry including the sodium, potassium, lithium, calcium, magnesium, and ammonium salts and the like which are prepared by methods well known in the art. The term also includes non-toxic acid addition salts which are generally prepared by reacting the compounds of this invention with a suitable organic or inorganic acid. Representative salts include the hydrochloride, hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, and the like.

The polypeptides meeting the foregoing conditions elicit antibodies in a mammalian host and are believed to be capable of forming a beta-turn structure that permits the polypeptide to immunologically mimick a desired conserved antigenic determinant site within a variable region of the carboxy-terminal half of the pilin protein. Preferably, the polypeptide is no more than about 60 amino acid residues long and contains in each defined antigenic determinant site at least one amino acid residue having a positively charged side chain.

One or more amino acid residue sequences meeting the foregoing conditions can be present as repeating units. Additionally, polypeptides containing one or more such amino acid residue sequences can be formed into relatively larger synthetic moieties by joining the individual polypeptides head-to-tail or by interpolypeptide cysteine disulfide bonds.

These polypeptides can be characterized as those including the amino acid residue sequences,

taken from left to right in the direction from the amino-terminus to the carboxy-terminus, of the formula

$$-X^1-X^2-X^3-X^4-X^5-$$

wherein each X designates an amino acid residue. Additionally, X^1 is an amino acid residue having a positively charged side chain such as HIS, LYS or ARG. X^2 and X^3 can be the same or different but are non-polar amino acid residues selected from the group consisting of LEU, PRO, TRP, PHE, VAL, ALA and ILE. Similarly, X^4 and X^5 can be the same or different but are polar, uncharged amino acid residues selected from the group consisting of SER, THR, CYS and GLY. The charges referred to above relate to ionic charges on the amino acid residues when those residues are present in an aqueous solution at pH 7.0. See for example, Lehninger, Short Course in Biochemistry, p. 37, Worth Publishers, Inc., New York, New York (1973).

Particularly preferred amino acid residue sequences within the above grouping, taken from left to right and in the direction from the amino-terminus to the carboxy-terminus, are

-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-;

-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-LYS-ALA-SER-ASP-ALA-LYS-; and

-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-PRO-VAL-THR-ARG-,

which sequences have been found to be conserved within the variable region of the carboxy-terminal half of pilin proteins from six different serotypes of N. gonorrhoeae strain MS 11, as described by Meyer et al., Proc. Nat'l Acad. Sci. USA (1984): 81:6110-6114, as well as the antigenically related variants thereof as defined hereinbelow.

More than one of the foregoing sequences can be present in the same polypeptide, usually spaced

from one another by a chain of other amino acid residues. In addition, the same polypeptide containing one or more of the foregoing amino acid residue sequences can also include a further amino acid residue sequence that immunologically mimicks an immunorecessive antigenic determinant site of a gonococcal pilin.

Another grouping of preferred polypeptides embodying the present invention, and free from intra-chain (intra-polypeptide) disulfide loops, is represented by polypeptides that are individually constituted by only one of the amino acid residue sequences specifically defined hereinabove, i.e.,

H-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-OH;

H-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-LYS-ALA-SER-ASP-ALA-LYS-OH; and

H-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-PRO-VAL-THR-ARG-OH.

Biochemical evidence from immunoassay and from analogy with conserved protein-protein interaction in solved X-ray crystallographic structures with differing sequences such as in the dimer contacts of oligomeric enzymes indicates that the conservation of protein-protein recognition does not require a strict conservation of sequence, for relatedness. While single amino acid residue changes may affect such recognition to a wide degree depending upon the nature of the substitution, in general terms the relatedness of two differing amino acid sequences with respect to protein-protein (and antigenic and/or immunogenic) recognition can be expressed in terms of seven basic amino acid parameters:

(1) hydrophobicity;

(2) evolutionary occurrence of changes in known sequences;

(3) size of side chain;

(4) charge and polarity;

(5) preference for turned secondary structure;

(6) preference for beta strand secondary structure; and

(7) preference for helical secondary structure.

To define the degree of sequence identity relevant to antigenic and/or immunogenic recognition, and thus antigenically related variants, a consensus matrix can be used to assign numerical values for each amino acid pair in the sequences being considered for relatedness. For the purposes of the present invention, the following consensus matrix, wherein the individual amino acid residues are designated by a one-letter code in the interests of conciseness, can be used:

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	7	-5	-1	-2	0	0	-1	2	-1	0	1	-2	2	-1	0	0	0	-3	-3	1
R	-5	10	0	-1	-3	2	-1	-5	5	-4	-4	5	-3	-2	-3	0	0	-1	-1	-4
N	-1	0	6	3	1	3	0	1	3	-2	-2	2	-1	-3	1	4	2	-3	0	-2
D	-2	-1	3	7	-2	1	4	0	0	-3	-3	0	-2	-4	0	1	0	-5	-2	-3
C	0	-3	1	-2	7	1	-2	1	0	0	0	-2	0	0	0	3	4	-2	2	0
Q	0	2	3	1	1	6	2	-1	4	0	0	2	0	0	0	1	3	-1	0	0
E	-1	-1	0	4	-2	2	7	-3	1	-3	-2	0	-1	-3	-2	0	0	-5	-3	-3
G	2	-5	1	0	1	-1	-3	8	-2	-3	-3	-2	-2	-5	2	3	1	-6	-2	-2
H	-1	5	3	0	0	4	1	-2	8	-1	0	4	0	0	0	1	2	0	2	-1
I	0	-4	-2	-3	0	0	-3	-3	-1	5	4	-3	2	2	-2	-2	0	0	0	4
L	1	-4	-2	-3	0	0	-2	-3	0	4	6	-2	4	3	-1	-2	0	1	0	3
K	-2	5	2	0	-2	2	0	-2	4	-3	-2	8	-1	-3	-1	0	0	-4	-2	-3
M	2	-3	-1	-2	0	0	-1	-2	0	2	4	-1	6	2	0	-1	0	0	-1	2
F	-1	-2	-3	-4	0	0	-3	-5	0	2	3	-3	2	7	-2	-3	0	4	3	2
P	0	-3	1	0	0	0	-2	2	0	-2	-1	-1	0	-2	7	2	1	-4	-1	-1
S	0	0	4	1	3	1	0	3	1	-2	-2	0	-1	-3	2	5	3	-3	0	-1
T	0	0	2	0	4	3	0	1	2	0	0	0	0	0	1	3	6	-2	1	0
W	-3	-1	-3	-5	-2	-1	-5	-6	0	0	1	-4	0	4	-4	-3	-2	9	2	0
Y	-3	-1	0	-2	2	0	-3	-2	2	0	0	-2	-1	3	-1	0	1	2	8	0
V	1	-4	-2	-3	0	0	-3	-2	-1	4	3	-3	2	2	-1	-1	0	0	0	5

Sequence comparison using the foregoing consensus matrix involves the determination of all possible alignments and the subsequent scoring of these alignments by the matrix. Two sequences are then aligned by computing the maximum match score from the consensus matrix. An alignment score in standard deviation units can be determined by taking the difference between the maximum matched score and the average maximum matched score for random permutation of the two sequences and then dividing by the standard deviation of the random score.

For the present purposes, a consensus matrix score greater than three standard deviations (approximately an average value of about 3 per residue) shows significant relatedness at a confidence level of more than 99.7%. This is a restrictive criterion since it gives a frequency of 0.005 for all 5-residue peptides and 0.0014 for all 13-residue peptides occurring in 2222 known protein sequences. Similarly, a consensus matrix score greater than two standard deviations (approximately an average value of abut 2 per residue) shows significant relatedness at a confidence level of more than 95.4%.

To determine relatedness for the purposes of the present invention, the consensus matrix score is calculated by ascertaining the matrix value for each aligned amino acid residue pair under consideration and then summing the individual values for each such pair. The obtained sum is then compared against the number of standard deviations signifying the desired confidence level. If the obtained sum is greater than the product of the selected number of standard deviations and the number of amino acid residue pairs under consideration, then the amino acid residue sequences being compared are antigenically related to the indicated confidence level.

For example, to ascertain the antigenic relatedness of the amino acid residue sequences

-LYS-TRP-PHE-CYS-GLY-

and

-ARG-ILE-PHE-CYS-GLY-

the consensus matrix yields the following values

	Value
-LYS- & -ARG- or K & R	5
-TRP- & -ILE- or W & I	0
-PHE- & -PHE- or F & F	7
-CYS- & -CYS- or C & C	7
-GLY- & -GLY- or G & G	8
Total	27

For antigenic relatedness at the 99.7% confidence level, the consensus matrix score must exceed the number of amino acid residue pairs under consideration times 3, i.e., 5 x 3 or 15. Inasmuch as 27 > 15, the desired antigenic relatedness is indeed present.

For the purposes of the present invention, antigenic relatedness among polypeptides within the scope of the invention preferably is present at least to about 95% confidence level, and more preferably to at least about 99% confidence level.

A mixture of the foregoing polypeptides, including those having antigenically-related regions as defined hereinabove, can also be used to make up a vaccine against or a diagnostic assay for a gonococcal infection, and/or an inoculum for raising antibodies.

Vaccines containing effective amounts of the present polypeptides induce production of antibodies in a sufficient amount to protect the vaccinated individual from infection with gonococcus and thus

prevent gonorrhea. Booster injections can be given if needed.

Thus, the word "vaccine" in its various grammatical forms is used herein in relation to the protection of a host mammal. The word "inoculum" in its various grammatical forms is used herein to describe a composition containing a polypeptide of this invention as an active ingredient used for the preparation of antibodies that immunologically bind to gonococcal pili. A vaccine and an inoculum may thus contain the identical ingredients, but their uses are different.

The polypeptides suitable as antigens or immunogens, or both, for the present purposes can be produced synthetically or by genetic engineering techniques, and can be in monomeric as well as multimeric forms for use in vaccines, inocula, or as diagnostics. When used in a vaccine or inoculum, the polypeptide may be used alone, as in the case of an oligomer or a multimer, or used linked to another carrier moiety as a conjugate. When used alone as an immunogen, a polypeptide of this invention typically contains a total of about 20 to about 35 amino acid residues. Shorter polypeptides are preferably linked to a carrier.

Particularly useful conjugate carriers include keyhole limpet hemocyanin (KLH), tetanus toxoid, poly-L-(LYS:GLU), peanut agglutinin, ovalbumin, soybean agglutinin, bovine serum albumin (BSA), human serum albumin, and the like.

The synthetic polypeptides utilized herein are preferably coupled to keyhole limpet hemocyanin (KLH) using the following well known method. The KLH carrier is first activated with m-maleimidobenzoyl-N-hydroxysuccinimide ester, and is subsequently coupled

to the polypeptide through a cysteine residue added to the amino-terminus or carboxy-terminus of the polypeptide by a Michael addition reaction, as described in Liu et al., Biochem. 80: 690 (1979).

A polypeptide of this invention may also be coupled to a carrier through different means, and may be coupled to carriers other than KLH as noted before. For example, a polypeptide may be coupled to a tetanus toxoid carrier through free amino groups, using a 0.04 percent glutaraldehyde solution as is well known. See, for example, Klipstein et al. J. Infect. Dis. 147: 318 (1983).

Cysteine residues added at the amino- or carboxy-terminii of the synthetic polypeptide have been found to be particularly useful for forming conjugates via disulfide bonds and Michael addition reaction products, but other methods well known in the art for preparing conjugates can also be used. Exemplary additional binding (linking) procedures include the use of dialdehydes such as glutaraldehyde (discussed above) and the like, or the use of carbodiimide technology as in the use of a water-soluble carbodiimide, e.g. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, to form amide links between the carrier and polypeptide.

As is also well known in the art, it is often beneficial to link the synthetic polypeptide to its carrier by means of an intermediate, linking group. As noted before, glutaraldehyde is one such linking group.

However, when cysteine is used for linking to the carrier, the intermediate linking group is preferably an m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS), also discussed before. MBS is typically first added to the carrier by an ester-amide

interchange reaction. Thereafter, the above Michael reaction can be followed, or the MBS addition may be followed by a Michael addition of a blocked mercapto group such as thiolacetic acid (CH_3COSH) across the maleimido-double bond. After cleavage of the acyl blocking group, a disulfide bond is formed between the deblocked linking group mercaptan and the mercaptan of the added cysteine residue of the synthetic polypeptide.

The choice of carrier is more dependent upon the ultimate intended use of the immunogenic polypeptide than upon the determinant portion of the immunogen, and is based upon criteria not particularly involved in the present invention. For example, if an inoculum is to be used in animals, as for the production of anti-polypeptide antibodies to be used to assay for the presence of gonococcal pilii, a carrier that does not generate an untoward reaction in the particular animal should be selected. If a vaccine against Neisseria gonorrheae is to be used in man, then the overriding concerns involve the lack of immunochemical or other side reaction of the carrier and/or the resulting immunogen, safety and efficacy--the same considerations that apply to any vaccine intended for human use.

The term "manufactured" as used herein means that the polypeptide molecule or polypeptide repeating unit has been built up synthetically by chemical means, i.e., chemically synthesized or by human-mediated biological means, e.g., by genetic engineering techniques. Thus, the manufactured polypeptides embodying the present invention are free from naturally occurring proteins and fragments thereof. The well-known solid phase chemical

synthesis in which blocked amino acid residues are added in a serial manner to obtain the desired polypeptide is the preferred method of synthesis, and is discussed in greater detail hereinbelow.

As mentioned hereinabove, polypeptides suitable for the purposes of the present invention can be synthesized by the well-known solid phase method. See, for example, Houghten et al., Int. J. Pept. Proc. Res. 16:311-320 (1980), and Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963), which disclosures are incorporated herein by reference. The solid phase method of polypeptide synthesis can be practiced utilizing a Beckman Model 990B Peptide Synthesizer, available commercially from Beckman Instruments Co., Berkeley, CA, U.S.A.

In preparing a synthetic polypeptide of this invention by the above solid phase method, the amino acid residues are linked to a resin (solid phase) through an ester linkage from the carboxy-terminal residue. When the polypeptide is to be linked to a carrier via a CYS residue, it is convenient to utilize that CYS residue as the carboxy-terminal residue that is ester-bonded to the resin.

The alpha-amino group of each added amino acid typically is protected by a tertiary-butoxycarbonyl (t-BOC) group prior to the amino acid being added into the growing polypeptide chain. The t-BOC group is then removed prior to addition of the next amino acid to the growing polypeptide chain. Reactive amino acid side chains are also protected during synthesis of the polypeptide. Usual side-chain protecting groups used for the remaining amino acid residues are as follows: O-(p-bromobenzyoxycarbonyl) for tyrosine, O-benzyl for threonine, serine, aspartic acid and

glutamic acid, and S-methoxy-benzyl for cysteine. Protected amino acids are recrystallized from appropriate solvents to give single spots by thin layer chromatography. Couplings are typically carried out using a ten-fold molar excess of both protected amino acid and dicyclohexyl carbodiimide over the number of milliequivalents of initial N-terminal amino acid. A two molar excess of both reagents can also be used. For asparagine, an equal molar amount of N-hydroxy-benzotriazole is added to the protected amino acid and dimethyl-formamide is used as the solvent. All coupling reactions are typically more than 99% complete by the picric acid test of Gisin, Anal. Chem. Acta, 58:248-249 (1972).

A portion of the resulting, protected, resin-bonded polypeptide (1 gram) is treated with two milliliters of anisole, and anhydrous hydrogen fluoride, 20 milliliters, is condensed into the reaction vessel at dry ice temperature. The resulting mixture is stirred at 4° for 1.0 hour to cleave the protecting groups and remove the polypeptide from the resin. After evaporating the hydrogen fluoride at a temperature of 4°C. with a stream of N_2, the residue is extracted with anhydrous diethyl ether three times to remove the anisole, and the residue is dried in vacuo.

The vacuum dried material is first extracted with 5% aqueous acetic acid (3 times 50 milliliters each) followed by extractions using 50% aqueous acetic acid (4 times 50 milliliters). The first extraction removes low molecular weight polypeptides and tyrosine that is used in some preparations to protect the CYS mercapto groups. The second extraction separates the free polypeptide from the resin. After dilution with water to a concentration

of 10-20% acetic acid, the resulting solution is lyophilized to provide a monomeric, unoxidized, polypeptide.

Polypeptide multimers can be prepared by bonding together the synthesized polypeptide monomers in a head-to-tail manner using the aforementioned solid phase method, i.e., one complete polypeptide sequence can be synthesized on the resin, followed by one or more of the same or different polypeptide sequences, with the entire multimeric unit thereafter being cleaved from the resin and used as described herein.

Alternatively, synthesized polypeptide monomers that contain added cysteine residues at both the amino- and carboxy-termini (diCYS polypeptides) can be bonded together by intramolecular, interpolypeptide cystine disulfide bonds utilizing an oxidation procedure to form an immunogenic polymer. The polymer so prepared contains a plurality of the polypeptides of this invention as repeating units. Those repeating units are bonded together by the above-discussed oxidized cysteine residues.

The presence of one or two terminal CYS residues in a polypeptide of this invention for the purposes of binding the polypeptide to a carrier or preparing a polymer is not to be construed as altering the amino acid sequence of the polypeptide repeating units from a sequence that immunologically mimicks an antigenic determinant site of a gonococcal pilin.

In a typical laboratory preparation, 10 milligrams of the diCYS polypeptide (containing amino- and carboxy-terminal cysteine residues in un-oxidized form) are dissolved in 250 milliliters of

0.1 molar ammonium bicarbonate buffer having a pH value of about 8. The dissolved diCys polypeptide is then air oxidized by stirring the resulting solution gently for a period of about 18 hours, or until there is no detectable free mercapton by the Ellman test. [See, Ellman, Arch. Biochem. Biophys. 82: 70-77 (1959).] The polymer so prepared is then typically isolated by freeze drying, redissolution and chromatographic purification.

Typical procedures for the preparation of fusion proteins by genetic engineering techniques are described by Berman, BioTechniques 1(4):178-183 (1983), Silhavy et al., Microbiological Reviews 47(3):313-344 (1983), and Young et al., Proc. Natl. Acad. Sci. USA 80:1194-1198 (1984).

The present vaccines and inocula include one or more of the polypeptides described hereinabove together with a pharmaceutically acceptable diluent such as physiological saline, phosphate-buffered saline (PBS), or other injectable liquid. Additives customarily used in vaccines or inocula may also be present, if desired. Illustrative of such additives are stabilizers such as lactose or sorbitol, and adjuvants such as aluminum hydroxide, sulfate or phosphate, an alum, or an alginate. Precipitated aluminum phosphate ($AlPO_4$) is a particularly suitable adjuvant for a vaccine, while complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) are preferred for use in inocula.

Vaccines and inocula of the present invention may be administered by injection, usually intramuscularly or subcutaneously, orally by means of an enteric capsule or tablet, as a suppository, as a nasal spray, and by other suitable routes of administration. For a human patient, a suitable dose

of the polypeptide depends, in part, upon the chosen route of administration and a number of other factors. Included among those factors are the body weight of the mammal to be immunized, the carrier when used, the adjuvant when used, and the number of innoculations desired to be used.

Individual innoculations for a human patient typically contain unit doses of about 10 micrograms to about 100 milligrams of polypeptide, exclusive of any carrier to which the polypeptide may be linked. If desired, a series of doses may be administered over a period of time for optimum immunity. Unit dosage forms of the vaccine can also be provided, if desired, containing the aforementioned amounts of the polypeptide.

In any event, the immunogen contained in a vaccine or an inoculum is present in an "effective amount," which amount depends upon a variety of factors as is well known in the immunological arts, e.g., the body weight of the mammal to be immunized, the carrier moiety used, the adjuvant used, the duration of protection sought, and the desired immunization protocol.

Whole antibodies, as well as substantially whole antibodies, raised to the polypeptides of this invention and antibody combining sites prepared from such antibodies constitute still another aspect of this invention. These molecules are collectively referred to as receptors.

Receptors are raised in mammals such as rabbits, goats, horses and the like by immunization using the inocula described hereinbefore. Immunization procedures are substantially the same as those used in vaccinations except that powerful adjuvants such as CFA and/or IFA that are not

acceptable for human use can be included in animal inocula.

Typical inoculum stock solutions are prepared with CFA, IFA or alum as follows: An amount of the polypeptide, synthetic polypeptide-conjugate or polymeric polypeptide sufficient to provide the desired, effective amount of polypeptide per inoculation is dissolved in PBS at a pH value of 7.2. Equal volumes of CFA or IFA are then mixed with the polypeptide solution to provide an inoculum containing polypeptide, water and adjuvant in which the water-to-oil ratio is about 1:1. The mixture is thereafter homogenized to provide the inoculum stock solution. When alum is used, about 200 micrograms of conjugate is absorbed onto about 4 milligrams of alum to prepare the stock inoculum.

Rabbits can be utilized herein to raise anti-polypeptide antibodies. When so used, the host rabbit is typically injected subcutaneously with an inoculum comprising 200 micrograms of a polypeptide conjugate (polypeptide plus carrier) emulsified in CFA; 200 micrograms of polypeptide conjugate in IFA; and 200 micrograms of polypeptide conjugate with 4 milligrams alum injected intraperitoneally on days 0, 14 and 21, respectively, of the immunization schedule. Each inoculation (immunization) consists of four injections of the inoculum. Mice may be immunized in a similar way using about one tenth of the above dose per injection.

Animals are typically bled 4 and 15 weeks after the first injection. Control pre-immune serum is obtained from each animal by bleeding just before the initial immunization.

Control inoculum stock solutions can also be prepared with keyhole limpet hemocyanin (KLH), KLH in

CFA or IFA, KLH-alum absorbed, KLH-alum absorbed-pertussis, edestin, thyroglobulin, tetanus toxoid, tetanus toxoid in IFA, cholera toxoid and cholera toxoid in IFA, and the like.

The efficacy of the above immunization procedure is typically determined by means of an ELISA in which the immunogenic polypeptide of this invention is used as the antigen to determine the amount of antibodies present in diluted sera obtained from the above bleeds. Sera that provide anti-polypeptide antibody titers (dilutions) of at least about 1:160 are considered useful in providing the antibodies of this invention. The typically utilized ELISA is described in greater detail in Bittle et al., Nature 298: 30-33 (1982), which is incorporated herein by reference.

Suitable monoclonal receptors, typically whole antibodies, may also be prepared using hybridoma technology as described by Niman et al., Proc. Natl. Acad. Sci., USA 80: 4949-4953 (1983), which description is incorporated herein by reference. Monoclonal receptors need not only be obtained from hybridoma supernatants, but may also be obtained in generally larger quantities from ascites fluid of mammals into which the desired hybridoma has been introduced. Production of monoclonal antibodies using ascites fluid is well known and will not be dealt with further herein.

A receptor of this invention binds both to the polypeptide to which it was raised and also to the corresponding pilin protein whose antigenic determinant site the polypeptide of this invention immunologically mimmicks. Thus, a polypeptide of this invention may be both an immunogen and an antigen.

The receptors of this invention are at least oligoclonal as compared to naturally occuring polyclonal antibodies since they are raised to an immunogen having relatively few epitopes as compared to the epitopes of an intact pilin molecule. Consequently, receptors of this invention bind to epitopes of the polypeptide while naturally occurring antibodies raised to a gonococcal pilin bind to epitopes throughout the pilin molecule.

The polypeptides, antibodies, and antibody combining sites provided by these polypeptides, and methods of the present invention may also be used for diagnostic tests, such as immunoassays. Such diagnostic techniques include, for example, enzyme immune assay, enzyme multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay (ELISA), radio-immune assay (RIA), fluorescence immune assay, either single or double antibody techniques, and other techniques in which either the antibody combining site or the antigen is labeled with some detectable tag. See generally Maggio, Enzyme Immunoassay, CRC Press, Cleveland, Ohio (1981), and Goldman, M., Fluorescent Antibody Methods, Academic Press, New York, N.Y. (1980).

An illustrative diagnostic system embodying the present invention to detect N. gonorrhoeae contains receptor molecules such as antibodies, substantially whole antibodies, or antibody combining sites, raised to a polypeptide of this invention. The system also includes an indicating means for signaling the presence of an immunoreaction between the receptor and the antigen. The indicating means allows the immunoreaction to be detected. When admixed with a body sample such as a cervical or urogenital smear, the receptor molecule immunoreacts

with the pilin antigen to form an immunoreactant, and the indicating means present then signals the immunoreaction.

One such exemplary embodiment is an immunofluorescent assay in which a cervical or urogenital smear is acetone-fixed to a plain microscope slide. An aliquot of antibodies raised in accordance with this invention, e.g., raised in rabbits, generally about 10 micrograms to about 500 micrograms, is incubated on the slides using well-known techniques.

After rinsing away any un-immunoreacted antibodies and blocking non-specific binding sites on the slide with a protein such as BSA, a second antibody, such as a goat-antirabbit antibody can then incubated on the test slide, if desired. The second antibody is labeled by being linked to a fluorochrome dye such as fluorscein isothiocyanate (FITC).

After this second incubation, any excess of the second antibody is rinsed off leaving any FITC-labeled goat-antirabbit antibodies that bound to the first antibodies on the test slide. Presence of the FITC-labeled antibodies may be detected using fluorescent microscopy and thereby signal the presence of a N. gonorrhoeae infection.

The use of whole, intact, biologically active antibodies for the receptor molecules is not necessary in many diagnostic systems such as the immunofluorescent assay described above. Rather, only the immunologically active, idiotype-containing, antigen binding and recognition receptor site; i.e. the antibody combining site, of the antibody molecule may be used. Examples of such antibody combining sites are those known in the art as Fab and $F(ab')_2$ antibody portions that are prepared by methods well known in the art.

Another diagnostic method of this invention is an ELISA assay. Here, a polypeptide antigen of this invention is bound on a solid support such as the walls of a microtiter plate. Non-specific binding sites on the microtiter well walls are thereafter blocked with a protein such as BSA. Un-bound polypeptide and BSA are removed from the microtiter well as by rinsing.

A body sample such as those above is admixed with an excess of an antibody of this invention in an aqueous solution, and the admixture is maintained for a time sufficient to form an immunoreaction between the antibody and any gonococcal pili antigen present. That liquid admixture is then mixed with the above-described polypeptide-bound solid support to form a second admixture containing solid and liquid phases. The solid/liquid phase admixture is maintained for a time sufficient for previously unreacted antibodies to immunoreact with the polypeptide antigen. The liquid phase is thereafter separated from the solid phase.

A solution of a second, labeled antibody that reacts with the first-named antibody is then admixed with the solid phase. An examplary second antibody is a peroxidase-linked goat anti-rabbit antibody where the first-named antibodies are raised in rabbits. The admixture formed from the solid phase and the second, labeled antibody solution is maintained for a time period sufficient to form an immunoreaction between the two antibodies. The solid and liquid phases are thereafter separated.

A solution containing a substrate for the enzyme such as hydrogen peroxide and a color-forming dye precursor such as o-phenylenediamine is thereafter admixed with the solid phase. The optical

density at a preselected wave length (e.g., 405 nanometers) may then by determined after a predetermined time period has elapsed and compared to the optical density of a control to determine whether the gonococcal antigen was present in the body sample.

The present invention is further illustrated by the following detailed examples.

EXAMPLE 1: Polypeptide Synthesis

A series of short synthetic polypeptides whose amino acid residue sequences correspond to small segments of the gonococcal pilin protein were synthesized accordig to the method of Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963), as modified by Houghten et al., Int. J. Pept. Proc. Res., 16:311-320 (1980), using a Beckman Model 990B Peptide Synthesizer (Beckman Instruments Co., Berkeley, CA, U.S.A.). The polypeptide designations and the location in the gonococcal pilin protein of their corresponding amino acid residue sequences are shown in Table 1, below.

TABLE 1

Synthetic Polypeptides Corresponding to Gonococcal Pilin Segments

Designation	Location[2]	Amino Acid Residue Sequence
44[1]	140-159	KEIDTKHLPSTCRDKASDA
GC4[1]	145-153	KHLPSTCRD
GC5[1]	144-159	TKHLPSTCRDKASDAK
GC6[1]	115-127	GSVKWFCGQPVTR

1 = polypeptides coupled to tetanus toxoid using the glutaraldehyde procedure described in Example 2.

2 = location corresponds to the amino acid residue positions of the gonococcal pilin protein sequence described by Meyer et al., Proc. Nat'l Acad. Sci. USA (1984) 81:6110-6114.

EXAMPLE 2: Polypeptide-Carrier Couplings

Polypeptide conjugation to tetanus toxoid carrier using the gluteraldehyde method was performed by admixing equal amounts of peptide and tetanus toxoid in PBS to final concentrations of 2 mg/ml. When difficulty in dissolving a particular peptide was encountered, the pH value of the produced admixture was raised to about 8.0.

A fresh glutaraldehyde working dilution was prepared prior to each coupling by diluting a 25% (w/v in PBS) glutaraldehyde stock solution 1:65 in ice cold PBS. The fresh glutaraldehyde solution was then added to the above obtained peptide-carrier solution at a ratio of 124 microliters to 1 milliliter, respectively. The resulting reaction composition was incubated with stirring overnight at room temperature.

After incubation, the reacation product was dialyzed for at least 6 hours against distilled H_2O, and then lyophilized.

EXAMPLE 3: Screening of Rabbit Sera for Anti-Polypeptide Antibodies

Rabbit anti-sera were screened for the presence of anti-polypeptide antibodies using an enzyme linked immunosorbent assay (ELISA). Polypeptide antigen made as described in Example 1, above, was adsorbed onto the walls of microtiter plate wells to provide solid phase bound target antigen.

To prepare solid phase bound polypeptide, 25µl of 0.1% (w/v) BSA/PBS containing approximately 5 picomoles of polypeptides was placed in a microtiter plate well and incubated at 37°C. until complete evaporation. Polypeptide antigen thus deposited was fixed to the solid phase by incubating 50µl of methanol in each well for 5 minutes at room temperature. After incubation, methanol was removed by inverted shaking and allowing the plates to air dry for 5-10 minutes.

Non-specific binding sites on the microtiter well walls were threafter blocked by incubating 50µl of 3% (w/v) BSA/PBS in each well for 4 hours at 37°C. in a humidified chamber. After incubation, excess BSA was removed by inverting and shaking the plates. Polypeptide bound to a solid support whose non-specific binding sites had been blocked was thus provided for use as target antigen.

To assay the rabbit sera for the presence of anti-polypeptide antibodies, an alioquot of each serum was serially diluted two-fold in 1% (w/v) BSA/PBS. Twenty-five microliters of each dilution was contacted with solid phase bound polypeptide by admixture in the appropriate microtiter wells prepared above. Contact was maintained by incubating the wells for about 16 hours at 37°C. in a humidified chamber, thus allowing any anti-polypeptide antibodies present in the serum dilutions to immunoreact with solid phase bound polypeptide target antigen. After incubation, the solid and liquid phases were separated by filling the wells with distilled water, inverting and shaking 10 times in seriatim.

To detect the presence of an immunorecation between anti-polypeptide antibodies and solid-phase

antigen, 25µl of goat anti-rabbit IgG labeled with peroxidase as an indicating means (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) were admixed in each well. After incubation for 1.5 hours at 37°C. in a humidified chamber, the wells were washed 10 times with distilled water as previously described. Fifty microliters of developer solution [55mg of ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate); Boehringer Mannheim]] and 100µl of 30% H_2O_2 dissolved in 100 ml of 0.1 sodium citrate (pH 4.2) were then admixed and incubated in each well for about 45-60 minutes at room temperature. The indicating reaction was stopped by admixing 50µl of 5% (w/v) sodium dodecylsulfate (SDS)/H_2O in each well. The amount of indicating reaction (color development) was quantitated by measuring the absorbance of each well at 414 nm.

Rabbit antisera demonstrating a 4-fold higher titer than the negative control were considered positive for the presence of anti-polypeptide antibodies. Antibodies that immunoreact with their inducing polypeptide were raised in rabbits to all the peptides shown in Table 1, above.

EXAMPLE 4: Western Blot Assay

The rabbit antisera were further screened to determine their ability to immunoreact with gonococcal pilin protein in a Western blot assay. Gonococcal pilin protein was isolated from the N. gonorrhoeae strain MS-11 (described in Meyer et al., supra) by the procedure outlined below.

N. gonorrhoeae MS-11 was plated and grown to confluency on GCB base medium (Diffco Co., Detroit, MI) in 15X100 mm petri dishes (American Scientific Products, McGaw, IL). The cultures from 50 dishes

were swabbed into 35 ml of 50mM ethanolamine, pH 10.5. The pili were sheared from the bacteria by vortexing for 30 seconds and immersing for 30 seconds in a 0°C. bath 4 times in seriatim.

The sheared pili were separated from the bacteria by centrifugation at 10,000 rpm for 20 minutes in a JA20 rotor (Beckman Instruments, Fullerton, CA). The supernatant containing pilin protein was then dialyzed against pili buffer (.15M NaCl, 0.05M Tris, pH 7.5) for about 16 hours. During dialysis a majority of the pilin protein present formed insoluble aggregates. These aggregates were subsequently isolated by centrifugation at 10,000 rpm for 15 minutes in the above-described rotor. The pilin protein pellet was then resuspended to a concentration of 1 mg/ml in 50mM ethanolamine, pH 10.5. The pilin was further isolated by eletrophoresis in a 15% SDS-polyacrylamide gel, and transferred to nitrocellulose (Schleicher & Schuel, Catalogue No. BA85, Keene, N.H.) using an electro-blotting apparatus (CBS Scientific, Del Mar, CA), with transfer buffer consisting of 25mM Tris, 192mM glycine, 20% methanol and 0.01% NaN_3.

The gonococcal pilin protein bound to the nitrocellulose solid support (Western blot) thus obtained was then used as target antigen in a Western blot assay to detect anti-pilin antibodies.

The rabbit anti-polypeptide antisera were diluted approximately 1:100 in 3% (w/v) BSA/PBS or BLOTTO [PBS containing 50 g/l nonfat dry milk, 0.1 ml/l antifoam A emulsion (Sigma Chemical Co., St. Louis, Mo.) and 1 ml/l thimerosal]. A Western blot was then incubated in 25 ml of each diluted antiserum for about 16 hours at 4°C. with constant agitation so as to contact the anti-polypeptide antibodies present

in the antiserum with the solid phase bound pilin protein.

After incubation, the blots were washed twice with 25 ml of TBS buffer (0.9% NaCl, 10mM Tris, pH 7.4) containing 0.5% octylphenoxy polyethoxy ethanol (Triton X100), washed three times with 25 ml of TBS alone, and then washed with 25 ml of 3% (w/v) BSA/TBS.

Anti-polypeptide antibodies bound to gonococcal pilin protein were detected by first reacting the blots with peroxidase labeled goat anti-rabbit IgG (Boehringer Mannheim) diluted about 1:2000 in 3% (w/v) BSA/TBS or BLOTTO for 16 hours at 4°C. with constant agitation. The blots were then washed as previously described and developed by incubation with 25 ml of the ABTS developing solution described in Example 3, above, for about 45-60 minutes.

The Western blot assay results indicate that all of the anti-polypeptide antisera raised in rabbits immunized with the polypeptides shown in Table 1 also contained antibodies that immunoreact with the gonococcal pilin protein.

EXAMPLE 5: Neisseria ELISA

The anti-polypeptide antibodies that immunoreacted with gonococcal pilin protein in the Western blot assay of Example 4, above, were examimed for their ability to immunoreact with whole bacteria used as solid phase target antigen in an ELISA. Eleven strains of Neisseria, including ten pathogenic gonococcal strains (Table 2, below) were obtained from J. Knapp, Neisseria Reference Lab, Seattle, WA. The strains were grown on GC chocolate agar at 35°C.. in a 5% CO_2 humidified incubator and confirmed by

Gram stain, oxidase reaction, colony morphology and sugar fermentation patterns as is well known in the art.

TABLE 2

Strains Tested

Strain NRL #	Description	
7122	N. gonorrhoeae	POMP 1*
8658	N. gonorrhoeae	POMP 2
7929	N. gonorrhoeae	POMP 3
6611	N. gonorrhoeae	POMP 4
5767	N. gonorrhoeae	POMP 5
8035	N. gonorrhoeae	POMP 6
5766	N. gonorrhoeae	POMP 7
8038	N. gonorrhoeae	POMP 8
8660	N. gonorrhoeae	POMP 9
1955	N. gonorrhoeae	WIII
9206	N. meningitidis B	

Neisseria antigen stocks were prepared by swabbing cultures propagated as described above into PBS (pH 7.4). The optical density of each bacterial suspension was then adjusted to 0.1 at 750 nm with PBS (pH 7.4), aliquoted and frozen.

Solid phase bound Neisseria antigen was prepared by adding 50µl of Neisseria stock suspension to microtiter ("Immulon 1" stripwells). The bacteria were pelleted from solution onto the walls of the wells by centrifugation for 5 minutes at 1000 g in a 4°C. tabletop centrifuge.

After centrifugation, the Neisseria antigen was crosslinked to the solid phase by admixing 200µl of 0.25% glutaraldehyde in PBS at 4°C. in each well. After a 5 minute incubation at 4°C., the wells were

washed 4 times with PBS. Non-specific binding sites were blocked by incubating 350µl of 5% BSA/PBS containing 0.05% polyoxyethylene(20)sorbitan monolaurate ("Tween 20") for 2 hours at 37°C. in each well. The BSA blocking solution was subsequently removed by aspiration, thus providing Neisseria as target antigen bound to the walls of microtiter wells.

Rabbit antipolypeptide antisera to be examined for anti-Neisseria activity were serially diluted two-fold in 1% (w/v) BSA/PBS. 200 Milliliters of each dilution were admixed in a well prepared as described above forming a solid/liquid phase immunoreaction composition. Contact was maintained by incubating the wells for 1 hour at 37°C., thus allowing the anti-polypeptide antibodies present in the serum dilutions to immunoreact with solid phase bound Neisseria antigen. After incubation, the solid and liquid phases were separated by subjecting the wells to three one-minute washes with PBS containing 0.05% "Tween 20."

Anti-polypeptide antibodies immunologically bound to the solid phase Neisseria were detected with a peroxidase-labeled goat anti-rabbit IgG indicating means obtained from Cappel, A Division of Cooper Biomedical, Malvern, PA. The goat anti-rabbit IgG was diluted 1:2000 in 1% (w/v) BSA/PBS and 200 µl were admixed in each well. The admixture was incubated for 1 hour at 37°C. thereby allowing immunoreaction between the goat anti-rabbit IgG antibodies and any rabbit anti-polypeptide antibodies present as bound to the solid phase Neisseria. After incubation the wells were washed five times for one minute each as described above.

The presence of goat anti-rabbit IgG antibodies bound to rabbit anti-polypeptide-Neisseria

immunoreaction products were detected by providing O-phenylenediamine as peroxidase substrate. Two hundred microliters of O-phenylenediamine developing solution (ODSI-Carpinteria, Carpinteria, CA) were admixed in each well and incubated for 30 minutes at room temperature. The color developing reaction was stopped by adding 50µl of 2N HCl to each well. The amount of inicating reaction present was immediately quantitated by measuring the absorbance of each well at 490nm using a Dynatech ELISA plate reader.

The above described ELISA was used to examine the anti-gonococcal pilin polypeptide antibodies for their ability to (1) differentiate between pathogenic and commensal Neisseria species; and (2) immunoreact with members of a representative panel of N. gonorrhoeae pathogenic strains.

In the first series of experiments, the anti-polypeptide antibodies were immunoreacted against the pathogenic N. gonorrhoeae strain POMP 1 serovar (NRL 7122) and the commensal N. meningitidis B strain (NRL 9206). The ability to differentiate between the pathogenic and commensal strains was then examined by calculating the ratio of the absorbance produced in the pathogenic strain ELISA versus the absorbance in the commensal strain ELISA. The results of examining the anti-polypeptide antibodies by this procedure are shown in Table 3, below.

TABLE 3

Rabbit Anti-N. Gonorrhoeae

Pilin Peptide Specificity Study

Peptide ID No.	Rabbit Antisera ID No.	ABS at 490nm N. gonorrhoeae (Strain 7122)	ABS at 490nm N. meningitidis B (Strain 9206)	Ratio[1]
--	9431[2]	0.037	0.136	--
--	9432[2]	0.068	0.013	--
44	T44	0.848	0.599	1.4
GC4	GC4-9307	0.661	0.324	2.0
GC4	GC4-9309	0.239	0.283	0.8
GC6	GC6-9310	0.274	0.119	2.3

1 = ratio of N. gonorrhoeae/N. meningitidis Absorbance Values; Antisera Dilution 1:3200.

2 = negative controls

As can be seen from the foregoing Table 3, antibodies induced by polypeptides made in accordance with the present invention, especially antibodies to polypeptides GC4 and GC6, as shown in Table 1, demonstrate significant immunospecificity for the pathogenic N. gonorrhoeae strain as opposed to the commensal N. meningitidis strain. The polypeptides of the present invention are suitable vaccine and inocula components because they direct the host's immune response towards disease-producing Neisseria species.

The second series of experiments utilizing the Neisseria antigen ELISA examined the ability of anti-polypeptide antibodies made in accordance with the present invention to immunoreact with members of a representative panel of N. gonorrhoeae pathogenic strains. The results of these experiments, shown in

Table 4, below, indicate that antibodies raised to the preferred polypeptides GC4 and GC6 have broad immunospecificity for pathogenic N. gonorrhoeae strains. Antiserum GC4-9307 contained a sufficient titer of anti-polypeptide antibodies to be able to recognize 9 out of the 10 pathogenic strains screened (i.e., all but NRL 8035) when used at a 1:1600 dilution and compared to the pre-immune control serum. Similarly, antiserum GC6-9310 recognized 7 out of the 10 pathogenic strains (i.e., all but NRL 8035, NRL 5766 and NRL 8038) at a 1:1600 dilution.

TABLE 4

Rabbit Antisera vs. Neisseria Panel

Strain	ABS at 490nm[1]		
NRL No.	GC4-9307	GC6-9310	9432[2]
7122	0.678	0.133	0.021
8658	0.541	0.229	0.003
7929	0.616	0.205	0.
6611	0.697	0.197	0.060
5767	0.659	0.191	0.098
8035	0.690	0.243	0.843
5766	>1.5	0.231	0.302
8038	1.069	0.169	0.220
8660	0.833	0.273	0.12
1955	0.550	0.220	0.084
9206	0.626	0.120	0.020

1 = antisera dilution 1:1600

2 = control antiserum

The range of pathogen strain immunospecificity (cross reactivity) demonstrated by

the results in Table 4, above, indicate that the polypeptides of the present invention induce broad spectrum antibodies. The ability to induce broad spectrum antibodies is a very desirable element in vaccine design because many pathogens, including N. gonorrhoeae escape destruction by a host's immune system through antigenic (strain) variation. The results in Table 4 therefore further demonstrate the utility of the polypeptides of the present invention for use as components in broad spectrum N. gonorrhoeae vaccines.

WE CLAIM:

1. A polypeptide which is smaller than a naturally occurring gonococcal pilin protein, which includes an amino acid residue sequence constituted by at least five amino acid residues and up to about 60 amino acid residues, and which is capable of immunologically mimicking a conserved antigenic determinant site within a variable region of the carboxy-terminal half of a gonoccoccal pilin.

2. A polypeptide containing no more than about 60 amino acid residues and including the amino acid residue sequence, taken from left to right in the direction from amino-terminal to the carboxy-terminal, of the formula

$$-X^1-X^2-X^3-X^4-X^5-$$

wherein X^1 is an amino acid residue having a positively charged side chain and is a member of the group consisting of HIS, LYS, and ARG, X^2 and X^3 are same or different non-polar amino acid residues that are members of the group consisting of LEU, PRO, TRP, PHE, VAL, ALA and ILE, and X^4 and X^5 are same or different polar, uncharged amino acid residues that are members of the group consisting of SER, THR, CYS and GLY; and pharmaceutically acceptable salts thereof.

3. The polypeptide in accordance with claim 2 wherein X^1 is HIS, X^2 is LEU, X^3 is PRO, X^4 is SER, and X^5 is THR.

4. The polypeptide in accordance with claim 2 wherein X^1 is LYS, X^2 is TRP, X^3 is PHE, X^4 is CYS, and X^5 is GLY.

5. The polypeptide in accordance with claim 2 and including the amino acid residue sequence -LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-.

6. The polypeptide in accordance with claim 2 and including the amino acid residue sequence

-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-
-LYS-ALA-SER-ASP-ALA-LYS-.

7. The polypeptide in accordance with claim 2 and including the amino acid residue sequence

-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-
-PRO-VAL-THR-ARG.

8. A polypeptide represented by the formula

H-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-OH

and a pharmaceutically acceptable salt thereof.

9. A polypeptide represented by the formula

H-THR-LYS-HIS-LEU-PRO-SER-THR-CYS-ARG-ASP-
-LYS-ALA-SER-ASP-ALA-LYS-OH

and a pharmaceutically acceptable salt thereof.

10. A polypeptide represented by the formula

H-GLY-SER-VAL-LYS-TRP-PHE-CYS-GLY-GLN-
-PRO-VAL-THR-ARG-OH

and a pharmaceutically acceptable salt thereof.

11. A peptide vaccine suitable for the prevention of gonorrhea which comprises a pharmaceutically acceptable diluent and a polypeptide defined by claim 1.

12. A peptide vaccine in accordance with claim 11 in unit dosage from wherein the polypeptide is present in an amount of about 10 micrograms to about 100 milligrams.

13. A method of immunizing against a gonococcal infection a susceptible patient which method comprises administering to the patient a pharmaceutically acceptable diluent and an effective amount of polypeptide defined by claim 1.

14. A diagnostic system suitable for assaying for Neisseria gonorrhoeae pilus protein which system comprises (a) receptor molecules

elicited in an aminal host by the polypeptide defined in claim 1 and (b) indicating means capable of signaling an immunoreaction of said receptor molecules with the pilus protein.

15. A receptor raised to the polypeptide defined by claim 1.

16. The receptor in accordance with claim 15 wherein the receptor is a whole antibody.

17. The receptor in accordance with the claim 15 wherein the receptor is an antibody combining site.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/00565

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

US CL. 260/112.5R; 260/112R; 424/88; 436/511;436/808;422/61

INT. CL.[3] CO7C 103/52;CO7G 7/00; A61K 39/00;GO1N 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	260/112.5R; 260/112R; 424/88; 436/511; 436/808; 422/61

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X P	US, A, 4,497,900 Published 5 Feb. 1985 Abram et al.	14-17
X	US, A, 4,188,371 Published 12 Feb. 1980 Weetall	14-17
A	US, A, 4,241,045 Published 23 Dec. 1980 Gaafar	1-17
A	US, A, 4,351,761 Published 28 Sep.1982 Gaafar	1-17
X	US, A, 4,186,182 Published 29 Jan.1980 Gaafar, et al.	11-17
X P	US, A, 4,461,838 Published 24 Jul.1984 Brinton, etal.	1-10
X	US, A, 4,330,623 Published 18 May1982 Karkhanis	15-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 JUNE 1985	21 JUN 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Robert R. Phillips

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE FIRST SHEET
(Not for publication)

A	US, A,	4,386,066 Published 31,May 1983 O'Rourke, et al.	1-17
X	N,	Chemical Abstract, Vol. 100, page 137079, Issued 1984, Hansen, et al.	1-10
X	N,	Chemical Abstract, Vol. 97, page 125478, Issued 1982, Judd	1-10
X	N,	Chemical Abstract, Vol. 98, page 196097, Issued 1983 Schoolnik, etal.	1-17
X	N,	Chemical Abstract, Vol. 96, page 50283, Issued 1982 Wong, et al.	1-10
A	N,	Chemical Abstract, Vol. 90, page 199948, Issued 1979 Swanson	1-17
A	N,	Chemical Abstract, Vol. 99, page 101900, Issued 1983 Judd	1-17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-10
II. Claims 11-13
III. Claim 14
IV. Claims 15-17

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.